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EP 0328403 A2 WO 92/18528 A1
Eur.J.Immunol. 1990,20,1619-1622 Int.J.Peptide Protein Res. 1991,37,27-32 J.Biol.Chem. 1988, 263, 1719-1725

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(54) **Annular antigen scaffolds comprising thioether linkages**

(57) Scaffolds of antigens are prepared by a convergent synthesis and coupling of soluble precursors comprising solubilizing groups. Cyclic peptide epitopes, known to be more effective immunogens than linear antigens because they are constrained to fewer conformations, are incorporated. In addition to the epitopes, linear T-haptens may be incorporated at either the C- or the N-terminus of the scaffold construct. The scaffolds constitute effective synthetic vaccines. The scaffolds are cyclised via a thioether linkage, the ring of which comprises from 3 to 10 lysine radicals, to which the epitope or antigen is bonded. The epitope or antigen is preferably an HIV V3 loop peptide, a malarial peptide, a GnRH peptide or a bacterial capsular polysaccaride.

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TITLE OF THE INVENTION
ANNULAR ANTIGEN SCAFFOLDS

BACKGROUND OF THE INVENTION

This patent disclosure relates to an annular antigen scaffold core or AASC for conjugation of antigens against which elicitation of immune responses in vivo is desirable. Thus, in one embodiment of this invention, an antigen scaffold is produced which elicits anti-human immunodeficiency virus immune responses, including the production of HIV neutralizing antibodies. In another embodiment, an immunogen for raising anti-malaria immune responses is produced. In another embodiment, bacterial capsular polysaccharides are conjugated to the AASC to produce a vaccine against pathogenic bacteria. In yet another embodiment, a chemosterilant is produced by elicitation of anti-gonadotropin releasing hormone immune responses.

It has now become standard practice in the art of vaccine production to prepare conjugates of poorly immunogenic epitopes and strongly immunogenic carriers for the purpose of enhancing the immunogenicity of the poorly immunogenic epitopes. For a recent review of conjugate vaccines, see Dintzis, R. Z., Pediatric Res., 32:376-356 (1992). Thus, capsular bacterial polysaccharides are conjugated to proteins, see for example US Patent 4,695,624, and peptidyl epitopes have likewise been conjugated, see for example, EP 0 467 700.

Immunogens directed at production of immune responses against human immunodeficiency virus (HIV) are under active development worldwide. Acquired immune deficiency syndrome (AIDS) and other diseases associated with HIV infection are reaching epidemic proportions on a global basis. This invention was made in response to the need for effective immunogens which are able to induce immune responses directed against the etiologic agent of these diseases.

Use of complete viral proteins, such as gp120 or gp160 has met with limited success as a vaccine candidate, as the subunit proteins appear to induce a number of immunological responses, not all of which are beneficial in preventing HIV infection.

Presentation of peptidyl epitopes displayed by the intact virus or its subunit proteins, has also been attempted. Thus, peptide epitopes have been conjugated to carrier proteins or they have been produced as fusion proteins with carrier proteins.

Of particular interest in this regard are HIV peptidyl epitopes derived from the V3 domain of gp120, referred to hereinafter as principal neutralizing determinant (PND) peptides. These are peptide epitopes against which HIV neutralizing antibodies are directed. A preferred subset of the PND sequences are those comprising the sequence Gly Pro Gly or Gly Pro Gly Arg [Seq. ID:1:]. This sequence is found in a great many isolates of HIV in the third variable region of the HIV envelope protein gp120, and appears to be part of an important epitope, proper exposure of which to mammalian immune systems results in induction of HIV neutralizing immune responses.

The above noted approaches hold promise, but the immunogens are complicated to produce, and successful HIV vaccines are not available. Those in clinical study are not conjugate vaccines and do not provide reproducibly high levels of neutralizing antibodies. Experimental conjugate vaccines are difficult to prepare and parameters of loading, sterility, and pyrogenicity are difficult to control. Replacement of the carrier protein with a low molecular weight moiety with known structure and composition to form a hybrid molecule will avoid these problems. Hybrid peptides are easier to prepare and are not subject to the same regulatory requirements as carrier protein conjugates.

A desirable method of producing an effective immunogen would be a completely synthetic route which yields a low molecular weight compound which is immunogenic. It is also desirable that the synthetic immunogen be capable of priming both B-cell and T-cell mediated immune responses, because HIV is known to multiply by cell-cell fusion (syncytia formation) and also to be present in the bloodstream where antibodies may bind to and neutralize the virus.

One disadvantage in prior techniques is that a large percentage of the mass of such immunogens is represented by the

carrier which, while enhancing the immunogenicity of the polysaccharide or peptidyl epitope to which it is conjugated, generally does not itself generate an immune response protective against the pathogen of concern.

One solution to this problem is the multiple antigen peptide, or MAP, advanced by James Tam [Vaccines 89, Cold Spring Harbor, p21-25; Tam, J.P., P.N.A.S., 85:5409 (1988); see also Prosnett, et al., J. Biol. Chem., 263:1719 (1988)]. The prototype MAP described by Tam consisted of a small peptidyl core matrix bearing seven dendritic lysl residues to which eight synthetic peptide antigens were attached to form an outer surface layer of acetylated synthetic peptides. According to that prototype, both the peptidyl core and the outer layer of peptides were synthesized as a single operation on a polymeric support by conventional step-wise, solid-phase (Merrifield) methods. Tam reported immune responses to fourteen different MAP constructs in rabbits and mice, and in eleven out of the fourteen constructs, the antibodies were specific for the native proteins from which the peptide epitopes were derived. While appearing to be effective, this method was limited in that desirable cyclic peptides could not be incorporated into the MAP. A multi-poly (DL-alanyl)-poly-L-lysyl conjugate of synthetic, cyclic lysozyme peptides was reported by Arnon et al., [P.N.A.S. USA 68 No.7:1450-1455 (1971)], but only disulfide bonded cyclic peptide conjugates were disclosed and the poly-lysine carrier was linear.

Since Tam's description of the MAP, several other workers have reported on the use of the MAP concept. Thus, EP 0 339 695 [published 11/2/89; see also Drijfhout, J. W., and Bloemhoff, W., Int. J. Peptide Protein Res., 37:27-32, (1991)], describes the separate preparation of the lysine core, the peptide antigens, and their subsequent coupling through disulfide bond formation. In addition, the incorporation of a solubilizing group, such as glutamic acid, between the lysine core and the antigenic peptide, was also described. This work added some flexibility and solubility to the MAP concept, but was limited in that coupling of peptidyl antigens through disulfides results in

the loss of antigen upon oxidation, resulting in diminished epitope presentation. Furthermore, the coupling efficiency of this system is subject to steric hindrance such that cyclic peptides are difficult to couple.

Compounds related to Tam's MAPs, referred to as "starburst dendrimers" have also been reported on [Roberts et al., Bioconjugate Chemistry 1, No.5:305-308 (1990); O'Sullivan, D. A., Chem. & Eng. News, August 16, 1993, pp20-24; Tomalia et al., Angew. Chem. Int. Ed. Engl. 29:138-175 (1990)]. These are three-dimensional, highly ordered oligomeric compounds which can mimic micelles and liposomes. These complex structures are reported to mimic biomolecules. However, their complexity is a major disadvantage in their use for vaccine production and they contribute substantial unnecessary mass to conjugates comprising them.

The concept of the MAP immunogen was extended to the development of anti-HIV-1 antigens by Nardelli, et al., [J. Immunol., 148:914-920 (1992); see also European Patent Application 89301288.0]. In that work, synthetic linear peptides from the V3 region of the gp120 of the IIIB, RF, and MN isolates of HIV-1 were used as the peptidyl antigens. Isolate specific antibody responses were raised by these immunogens in rabbits and mice.

In EP A 0 328,403, dendritic multiple antigen peptides, using linear HIV peptides, were prepared.

In WO 9222583, a concept similar to the MAP was used to produce tri- and tetra-valent mono-specific antigen-binding proteins consisting of 3 or 4 Fab fragments bound to a connecting structure for treating cancer.

In WO 9218528 (published Oct. 29, 1992), a branched peptide MAP was described wherein a lysine core was linked to peptide epitopes with the interposition of a spacer comprising 1-20 glycine residues. In PCT/US90/02039, a MAP in which malaria antigens were bound to a core molecule were disclosed.

In WO93/03766 (published 4 March 1993), a MAP including a peptide from the V3 loop of gp120, from cysteine 303 to

cysteine 338 of HIV-1, was disclosed. The only type of circularized peptide envisioned, however, were disulfide bonded peptides (see WO93/03766, page 9, lines 29-33). Such peptides are notoriously labile, and in the physiological mileu, are likely to spend a large proportion of the time in a linear conformation.

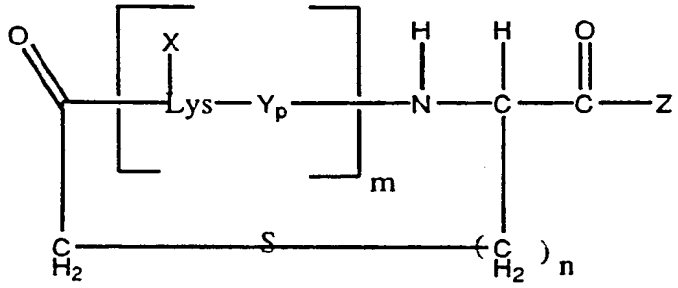
While the MAP concept has been applied to linkage of linear antigens or disulfide-bonded cycles, it has not been applied to stably cyclic antigens. According to Satterthwait, A.C. et al. [Phil. Trans. R. Soc. Lond. B323:565-572 (1989); Bulletin of the World Health Organization, 68 (Suppl.):17-25 (1990)], small conformationally constrained cyclic peptides were reported to be useful anti-malaria immunogens. Lacroix, et al. [V Conf. Int. SIDA, Ed. Morrisset, R, Abstract W.B.P.110, Montreal (June 1989)], compared the performance of cyclic and linear peptides covering an immunodominant region of HIV-1 and HIV-2 transmembrane glycoproteins in an ELISA. They concluded that the cyclic peptides were superior antigens than the linear counterparts, and that they can form the basis of highly sensitive and specific tests for HIV-antibody detection.

In the instant invention, an annular antigen scaffold core (AASC) is used to prepare an antigen scaffold with enhanced solubility and coupling efficiency using a convergent synthesis technique. A wide variety of epitopes may be conjugated to the AASC of this invention. These applications are exemplified by coupling of novel stably cyclic HIV principal neutralizing determinant (PND) peptides, GnRH peptides, type-specific bacterial capsular polysaccharides, and malaria antigens to produce, respectively, an immunogen to raise anti-HIV immune responses, a chemosterilant, a vaccine against pathogenic bacteria, and an immunogen for inducing anti-malaria immune responses.

SUMMARY OF THE INVENTION

An annular antigen scaffold core (AASC) for attaching and presenting antigens to the immune system is prepared by convergent synthesis and coupling of soluble precursors comprising solubilizing groups and groups enhancing the efficiency of coupling. Linear and

cyclic peptide epitopes, and preferably the cyclic epitopes which are more effective immunogens than linear antigens because they are constrained to fewer conformations, are conveniently incorporated. In addition to cyclic B-haptens, linear T-haptens can be incorporated at either the C- or the N-terminus of the peptide scaffold. The peptide scaffold may be represented by the general formula:



wherein:

S is a sulfur atom, forming a thioether bond;

X is an epitope with each X being linked through the α- or ε-amino group of lysine, with each X being the same or different;

Y is a linker;

Z is a moiety linked to the antigen scaffold, such as ß-alanine or a peptide providing a T cell epitope, a lipopeptide which may provide an adjuvant effect, or another moiety performing a carrier function;

p is 0 to 5;

m is 3 to 10; and

n is 1 to 10.

X may be any epitope against which elicitation of an immune response is desired. Thus, X may be a linear or cyclic human immunodeficiency virus principal neutralizing determinant. X may be a malaria antigen. X may be a type-specific bacterial capsular polysaccharide. Where X is a gonadotropin releasing hormone (GnRH, also known as luteinizing hormone releaesing hormone or LHRH), or an analog thereof, the scaffold induces anti-GnRH antibodies. This

embodiment of the invention acts as a chemosterilant. Peptide scaffolds of this invention constitute effective synthetic vaccines.

DETAILED DESCRIPTION OF THE INVENTION

An annular antigen scaffold, hereinafter referred to as an AAS, of this invention is made by:

a) Synthesizing an annular multi-lysine core, referred to herein as an annular antigen scaffold core (AASC), optionally incorporating solubilizing or sterically unhindered groups and purifying this component;

b) Isolating or synthesizing an antigen optionally incorporating a terminal group of low steric hindrance and purifying this component; and

c) Coupling the purified core component of step (a) with the purified antigen of step (b), and purifying the AAS thus formed. The order of the foregoing steps need not be precisely as described above.

The multi-lysine core may consist of a matrix of a few or many lysines which may be either the L or D isomer. Practically, however, a tri- or tetralysine core is generally sufficient, easily and reproducibly synthesized, and capable of being conjugated to four antigenic molecules. The lysine core is prepared by standard solid phase peptide synthetic methods. For example, a resin functionalized with a ß-Ala is reacted with the free carboxy group of cysteine. Several lysines are linked through standard solid phase chemistry to form peptide bonds (using either the alpha or epsilon amino group of each lysine to extend the chain) to form, for example, a tetralysine oligopeptide. The cysteine at the carboxy terminus is included to provide a sulfhydryl for ring closure to form an annular structure. The amino terminus of the peptide is bromoacetylated to enable reaction with the free sulfhydryl. Once the oligopeptide is liberated from the solid phase synthesis resin and protecting groups are removed, the peptide is cyclized to form a circular (annular) peptide through a thioether linkage. This circular peptide forms the AASC of this invention.

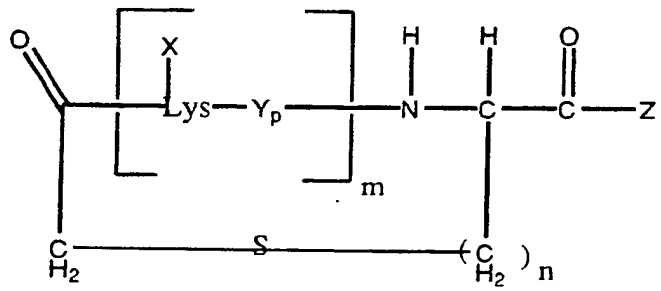
Steric hindrance may be reduced by coupling one or more non-steric groups to the free amino group of each of the lysines monomers used to extend the multi-lysine peptide chain. Thus, for example, each lysine is acylated to a glycine residue and then added to the nascent chain. In this manner, access of bulky epitopes to the core and conjugation to the core is facilitated. In this way also, following cyclization and once the annulus is fully loaded with antigens, the appended epitopes are fully exposed to the immune system. Thus, groups of low steric hindrance, such as glycine or a similar compound of formula:

NH_2-$(CH_2)_x$-COOH,

wherein x is 1-10, and is preferably glycine (x = 1), are appended on the AASC peptide at the site to which each antigen molecule is to conjugated.

We have discovered that it is desirable to purify the lysine core before reacting the core with antigenic epitopes. Starting with a purified core eliminates incompletely synthesized scaffold cores. To enhance solubility of antigen scaffolds, it is advantageous to add hydrophylic groups, such as serine, onto the nascent lysine core, for example, between each lysine and the sterically unhindered group such as glycine, before it is cleaved from the resin. It is important not to utilize groups having functionalities which potentially can react with free amino groups (such as carboxyllic acid groups on glutamic or aspartic acid). While many such solubilizing groups may be added, a point is reached where such groups net little increase in the solubility of the core while adding mass to the core that does not productively contribute to the desired immune response. In our experience, addition of from one to five serines is sufficient to render the antigen scaffold soluble even when hydrophobic epitopes are conjugated. Naturally, the more hydrophobic the epitopes and the larger the number of conjugated epitopes, the more solubilizing groups will be needed.

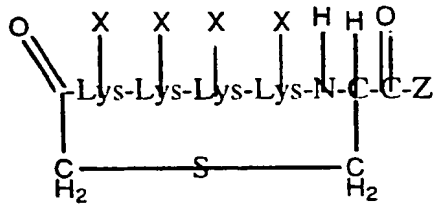
The AASC of this invention may be represented by the formula:



wherein:
S is a sulfur atom, forming a thioether bond;
X is an epitope with each X being linked through the α- or ε-amino group of lysine, with each X being the same or different;
Y is a linker;
Z is a moiety linked to the antigen scaffold, such as ß-alanine or a peptide providing a T cell epitope, a lipopeptide which may provide an adjuvant effect, or another moiety performing a carrier function;
p is 0 to 5;
m is 3 to 10; and
n is 1 to 10.

The linker Y may be a lower alkyl chain of one to five carbons. Y may also be NH_2-$(CH_2)_x$-COOH, wherein x is 1-10, and is preferably glycine (x = 1). Y may also be an unusual amino acid such as ß-alanine. Essentially, any linking group which provides some spacing between the lysine residues of the AASC but which does not otherwise alter the properties of the core may be used.

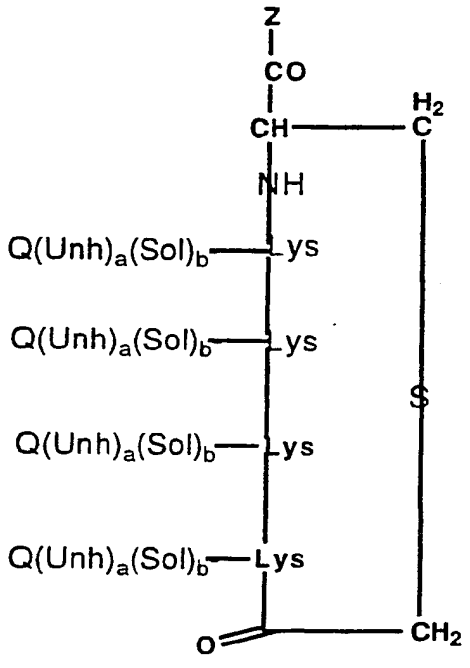
In one embodiment of the invention, the AASC has the structure:



wherein:
S is a sulfur atom, contributed by the cysteine shown, forming a thioether bond;
X is an antigen with each X being the same or different;
Z is a moiety linked to the antigen scaffold, such as ß-alanine, a lipopeptide adjuvant, or another T-cell epitope.

The group "X" may be further defined as being comprised of the groups $(Unh)_a(Sol)_bQ$, wherein:
Unh represents sterically unhindered groups;
Sol represents hydrophilic, solubilizing groups;
Q represents an antigen;
a is 0-5; and
b is 0-5.

Thus, in particular embodiments of this invention, the AAS has the formula:



wherein:
Z is a moiety linked to the antigen scaffold, such as ß-alanine, a lipopeptide adjuvant, or another T-cell epitope;
Unh represents sterically unhindered groups;
Sol represents hydrophilic, solubilizing groups;
Q represents an antigen;
a is 0-5; and
b is 0-5.

The AASC provides a compact and easily synthesized structure for the scaffold of this invention which, once the antigens have been linked, can display the antigens surrounding the annulus, thus essentially obscuring the presence of the core from the immune system as completely as possible. In this fashion, minimal immune responses against the core structure are elicited.

Once the core has been synthesized as described above, it is purified according to standard peptide purification procedures known in the art. A particularly preferred method is to purify the core by reverse-phase high performance liquid chromatography (RPHPLC) as shown in the examples below.

Linkage of antigens to the AASC may be accomplished in a variety of different ways. In one method, the free amino groups of the core are bromoacylated, or maleimidated. The core may then be reacted with antigens bearing free sulfhydryl groups, as in 6-D-Cys-GnRH, with epitopes which have been thiolated, for example by provision of a cysteine anywhere in the antigen molecule that does not interfere with its immunogenic epitopes, or reaction with a reagent such as N-acetyhomocycteine thiolactone to thiolate free amines on the antigen. Alternatively, free amino groups of the core may be thiolated, for example by reaction with N-acety homocysteine thiolactone, and the antigens may be bromoacylated or maleimidated. Essentially any method where nucleophillic and electrophillic groups are provided on the reacting partners is sufficient to achieve linkage of epitopes and the AASC. As a result of these diverse linking strategies, linkages including but not restricted to the following, are produced:

(a) Direct coupling through an amide linkage to yield peptide bonds, as in coupling of active esters with free amines.

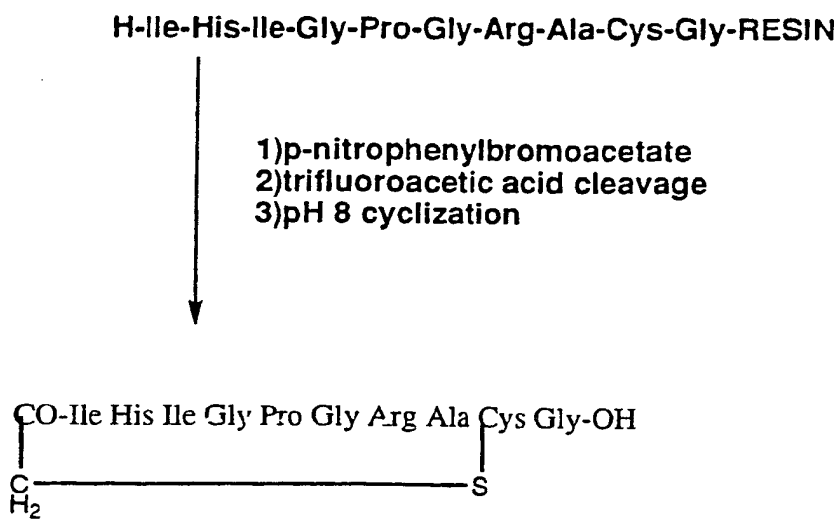
(b) Coupling through a linker, as in the use of a reagent such as succinic anhydride to produce a linker of formula $-NHCO(CH_2)_nCO-$, wherein this "n" may between one and five.

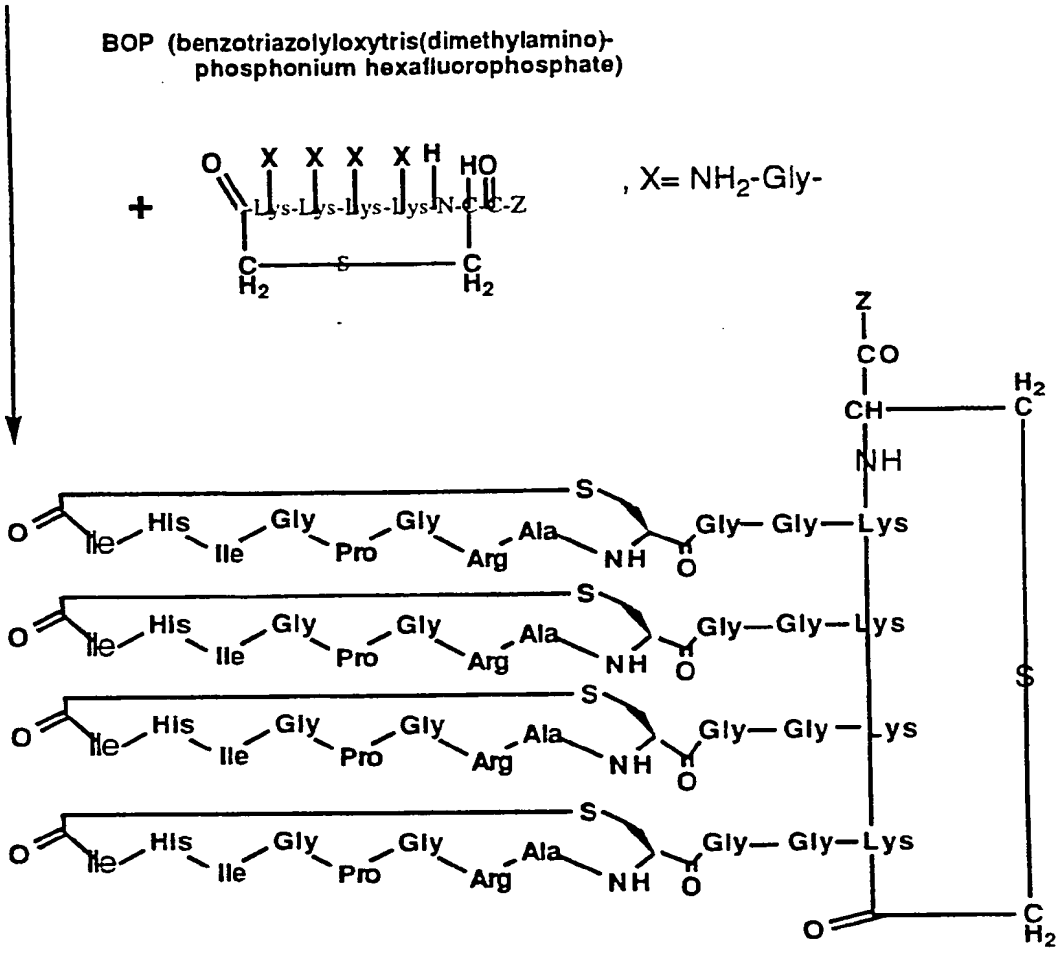
(c) Coupling through a linker, as in the use of a bromoacylating agent and a thiol linkage, to produce, for example, a linker such as $-NH-CO(CH_2)_n-S-$ between the antigen and the AASC. As noted above, however, any of a variety of linkages may be used to produce the conjugate annular antigen scaffold of this invention and this discussion is meant to be mearly illustrative, as are the examples which follow.

In one embodiment of this invention, the peptide is a novel, stably cyclic human immunodeficiency virus (HIV) principal neutralizing determinant (PND) peptide as disclosed herein and claimed

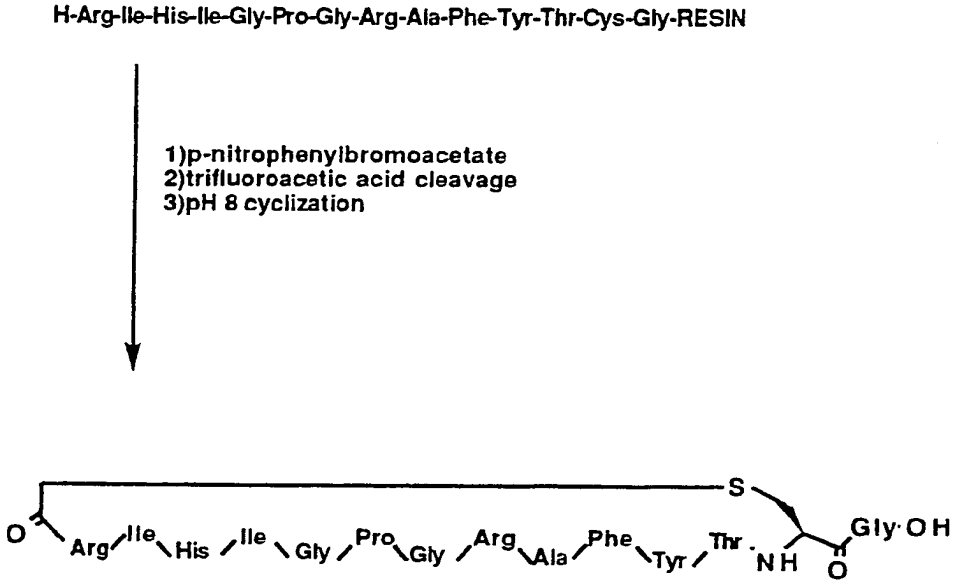
in application USSN , (Attorney docket number 19009, concurrently filed herewith). According to this embodiment of the invention, antigenic scaffolds are prepared by coupling purified cyclic HIV PND epitopes to a purified AASC. Thus, a tetraepitope scaffold which could be purified by HPLC and verified by electrospray mass spectrometry is provided as an example. The process involves synthesizing a linear HIV PND peptide on a solid resin support, bromoacetylating the N-terminal amine, deprotecting and cleaving peptide from the resin (using TFA for Fmoc chemistry or HF for Boc chemistry), cyclizing the peptide at about pH 8 between the bromoacetyl and the sulfur on a cysteine to form a cyclic thioether, and coupling the cyclic thioether to the AASC directly, via a Gly-Gly peptide bond for the purpose of eliminating steric hindrance and racemization problems. In another example of this embodiment, the solubility of the scaffold is enhanced by introducing a hydrophilic linker of either glycine or serine between the scaffold and the peptide epitope. These syntheses are shown in Schemes I-III respectively:

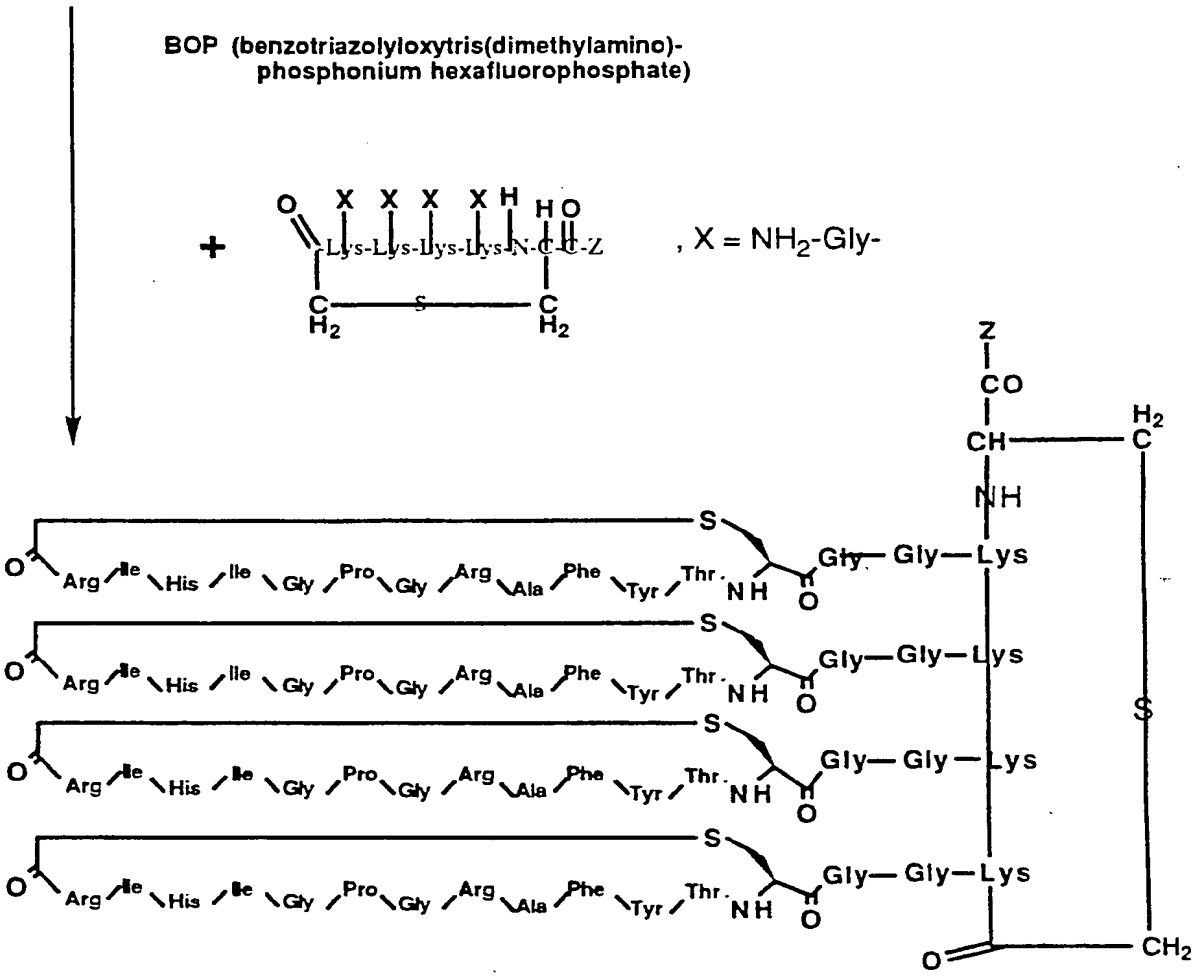
Scheme I, [SEQ.ID:2:]:



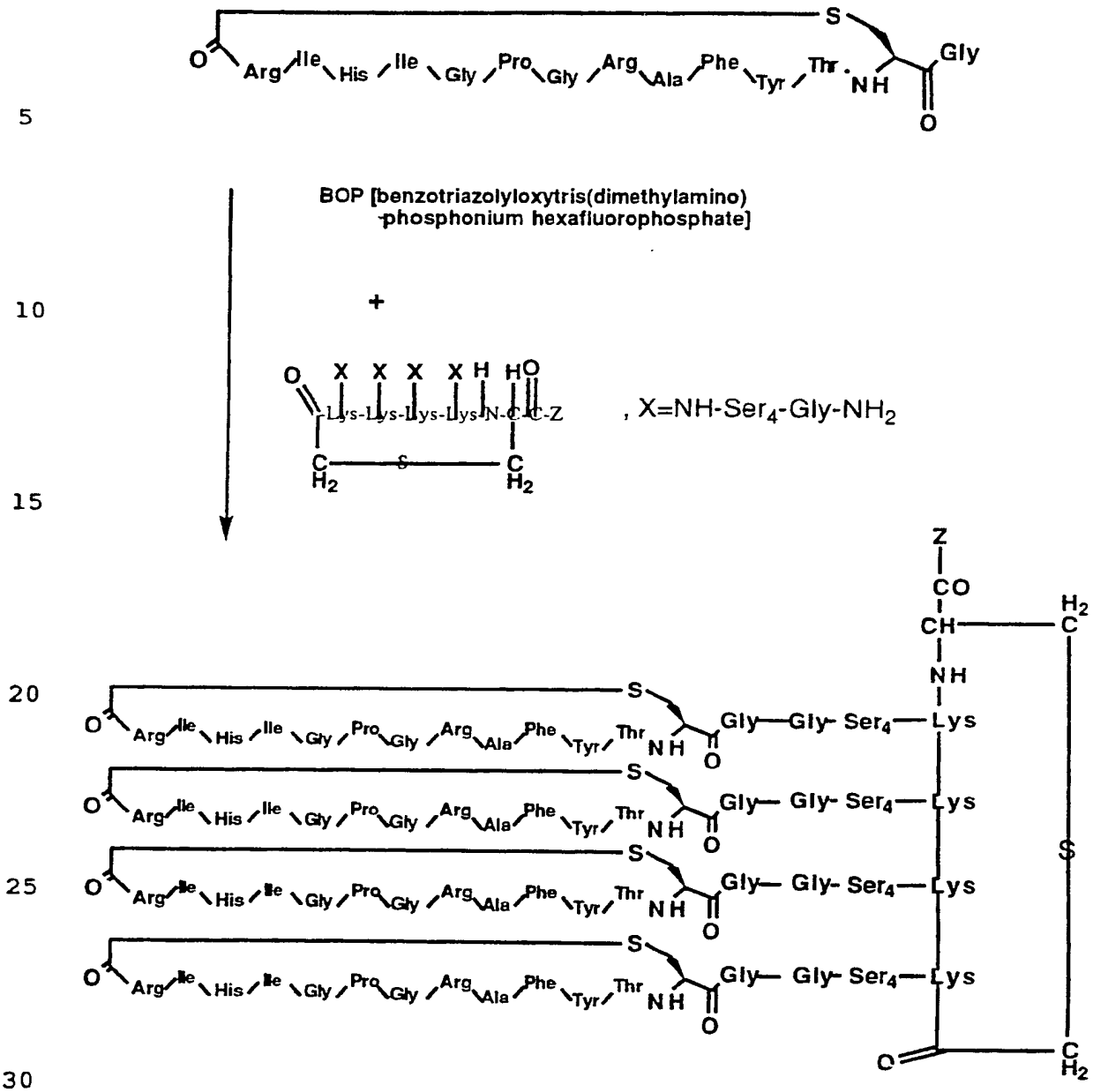


Scheme II, [SEQ.ID:3:]:



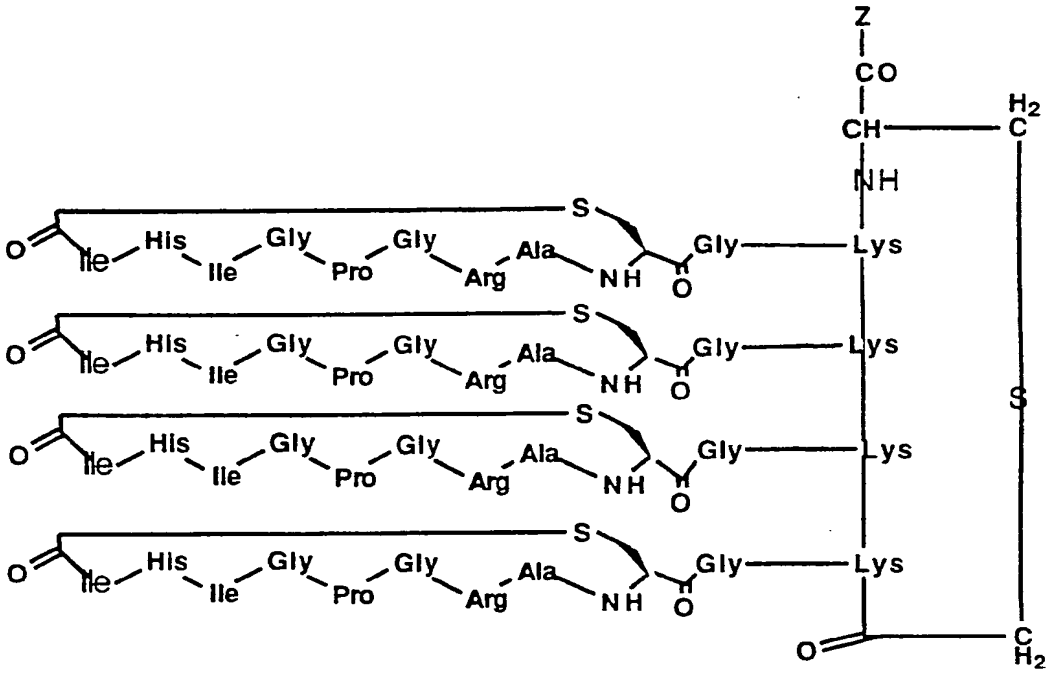


Scheme III, [SEQ.ID:3:]:

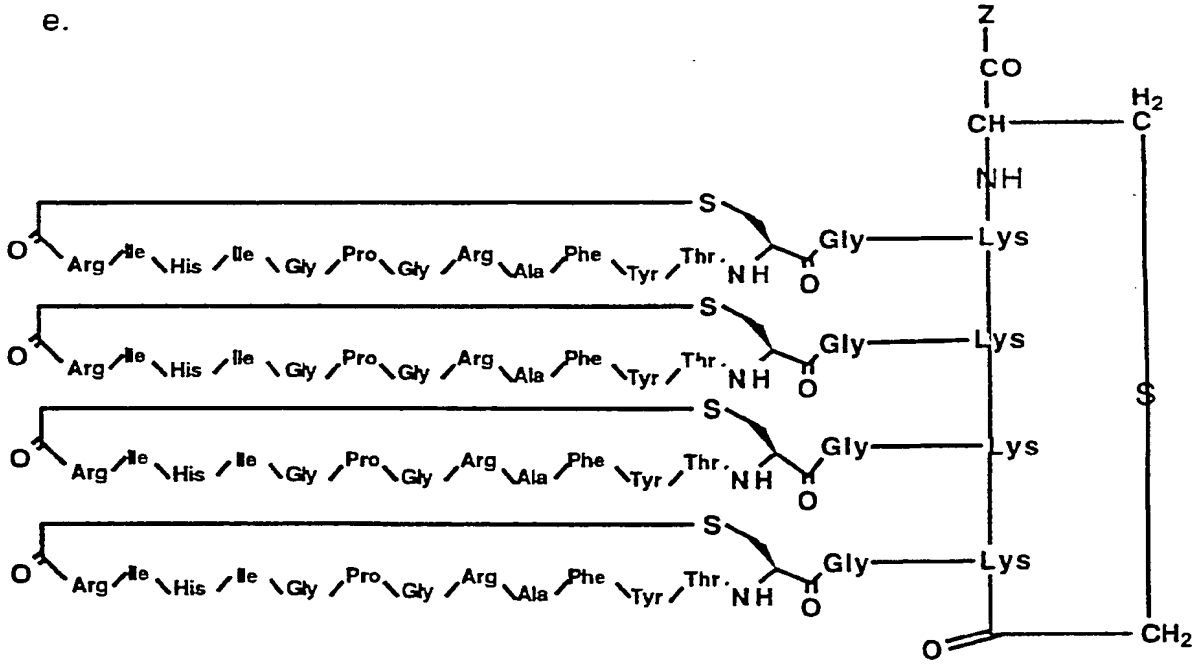


Other embodiments of the invention include the compounds:

d.



e.



In another embodiment of this invention, the AASC is conjugated with GnRH peptides. The immunogen so produced raises antibodies which deplete GnRH in the biological system. As a result, the immunogen acts as an effective chemosterilant. According to this embodiment of the invention, any of the known GnRH analogs may be used as the antigen. An example of a synthesis of this embodiment is provided wherein 6-D-Cys-GnRH is coupled to the AASC.
As a particular example of how to make and use the AASC of this invention, gonadotropin releasing hormone epitopes are described and their activity within the AASC context is demonstrated.

A number of publications have disclosed attempts to provide GnRH chimeric toxins for the purpose of destroying GnRH receptor expressing cells in the pituitary. The disclosure of WO 93/113777, which disclosed chimeric molecules of GnRH or analogs thereof and cytotoxins in which GnRH receptor binding ligands were directly linked to a toxin functionality, and of WO 92/22322 and WO 90/09799, are hereby incoroporated by rereference for what they teach in regard to GnRH and its analogs.

Naturally occuring GnRH has the formula: Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, [SEQ ID:4:] (Pyr is pyroglutamic acid), a decapeptide (M_r) which stimulates the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the anterior pituitary. LH and FSH stimulate the production of sex hormones by the gonads. This compound, and analogs thereof, in which linkage to the AASC is enabled by inclusion of easily derivatized amino acids at the naturally occurring Gly at the six position, may be used to make immunogens built on the AASC of this invention.

The GnRH antigen may be defined with reference to the following structure [Seq. ID:5:]:

Q-Ser-Tyr-W-X-Arg-Y-V
|
L_1

L2
AASC

wherein:
Q is PyroGlu-His-Trp, N-acetyl-4-Cl-Phe^{1,2}-Trp or 3-indolylpropionyl;
AASC is annular antigen scaffold core to which the GnRH peptide is linked;
W is a D or L amino acid with a pendant linking functionality such as cysteine, or lysine;
X is Leu or Nle;
Y is Pro or 4-hydroxy-Pro; and
V is Gly, NH_2, D-Ala-NH_2, NH-Et, NH-Pr or Arg-Gly-NH_2.
L_1 and L2 are portions of the linker as described above.

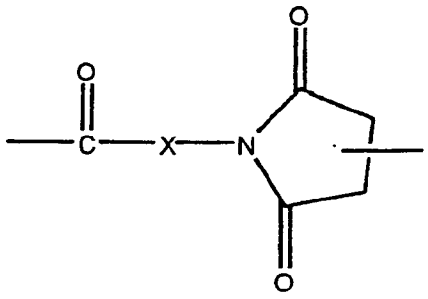
Preferred compounds of the instant invention are realized in the following structure [Seq. ID:6:]

PyroGlu-His-Trp-Ser-Tyr-W-Leu-Arg-Y-V
1 2 3 4 5 6 7 8 9 10
L_1
L2
AASC

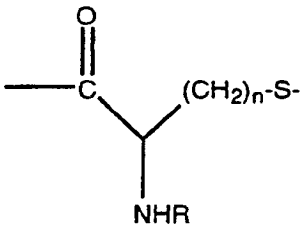
where the decapeptide is GnRH with the normal 6-position amino acid (W=Gly) deleted and replaced by W=D-Lys, D-Orn, or D-Cys.

D-Lys^6-GnRH or D-Cys^6-GnRH are preferred GnRH epitopes to link to the AASC. It will be recognized that variations of these GnRH analogs that can induce antibody production to prevent binding of GnRH to the GnRH receptor of the pituitary will be useful in this invention. All that is required is that the 6-position amino acid possess a group for binding to the linking group and that the remainder of the peptide retain the ability to induce an anti-GnRH immune response.

L_1 and L_2 may include any of the linkages described above. In addition, L_1 and L_2 may comprise, independently, the groups:



or



wherein:
X is C_1-C_5 alkylene, phenyl or C_5-C_6 cycloalkylene;
R is C_1-C_3 alkanoyl; and
n is 1 or 2.

In yet another embodiment of this invention, the AASC is conjugated with malaria antigens. Small constrained cyclic-peptides are known in the art [Satterthwait A.C. et al., Phil. Trans. R. Soc. Lond. B323:565-572 (1989); Bulletin of the World Health Organization, 68 (Suppl.):17-25 (1990)], and have been reported to be useful anti-malaria immunogens which elicit sprorozoite-specific neutralizing antibody. A fully effective vaccine is produced by addition of vaccine components specific for the schizont and merozoite stages of the parasite. This embodiment of the invention is provided by coupling peptides such as: Cys-^{1}Asn-Pro-^{1}Asn-Ala-^{2}Asn-Pro-^{2}Asn-Ala-^{3}Asn-Pro-^{3}Asn-Ala-NH_2 [SEQ.ID:7:], or Cys-^{1}Asn-Ala-^{1}Asn-Pro-^{2}Asn-Ala-^{2}Asn-Pro-^{3}Asn-Ala-^{3}Asn-Pro-NH_2 [SEQ.ID:8:], wherein the superscript numerals indicate bridges between like numbered asparagine residues, to the AASC of this invention.

For conjugation of type-specific bacterial capsular polysaccharides, the capsular polysaccharide of Haemophilus influenzae type b, or of Streptococcus pneumoniae, available from ATCC, and

activated for conjugation according to methods known in the art (see US Patents 4,695,624 and 4,830,852, herein incorporated by reference). Thus, a capsular bacterial polysaccharide is reacted with a bis electrophile such as carbonyldiimidazole, followed by reaction with a diamine such as 1,4-butanediamine. The amine functions on the polysaccharide are then bromoacetylated [see Marburg et al., J. Am. Chem. Soc. 108:5282-5287 (1986)]. The bromoacetylated polysaccaride can be reacted with thiolated AASC, prepared, for example, by activation of the AASC free amines using N-acetylhomocysteine thiolactone or a similar thiolating reagent. In this embodiment, the AASC replaces more bulky immune carriers such as OMPC, the outer membrane protein complex of Neisseria meningitidis b.

In a preferred embodiment of this invention, in addition to the unhindered groups and solubilizing groups, the group Z is appended which has adjuvant activity. To this end, lipopeptide moieties may be attached at the position shown in the structure for AASC. Lipopeptides useful in this invention include synthetic lipopeptide analogs of the E. coli or S. willmorei lipopeptide. In general, these synthetic lipopeptide analogs are triacyl-glyceryl-cysteinyl-seryl-serine, and are preferably (Palmitoyl)$_3$glycerylCys Ser Ser.

According to this embodiment of the invention, an AASC HIV PND peptides is prepared as disclosed above. The AAS is then acylated by the (Pam)$_3$Cys-OH at the carboxy terminus, care having been taken to ensure that all other free amines in the scaffold are appropriately protected. This is accomplished by attaching the antigens to the core and then providing a free amino anchor on the core's carboxy terminus for attachment of the lipopeptide (see Example 6). A synthetic hybrid-immunogen comprising a triacyl-glycerol cysteinyl seryl serine or cysteinyl- asparaginyl-seryl-glycyl-glycyl-serine [SEQ.ID:9:] moiety linked to a cyclic HIV principal neutralizing determinant peptide scaffold may be prepared by separately preparing each subportion, and then linking the two subportions. A tripalmitoyl-glycerol linked cysteine (Pam)$_3$Cys-OH moiety may be prepared according to methods disclosed in the literature. Thus, the methods of

Wiesmuller et al [Hopper-Seyler's Z Physiol Chem 364, 593-606 (1983)], Jung et al [Liebigs Ann. Chem. 1608-1622 (1983)], Bessler et al, [J. Immunol 135, 1900-1905 (1985)], Jung et al [Angew. Chem. Int. Ed. Engl 24, 872-873 (1985)], and Jung, EP 0431 327 AI may be used to prepare $(Pam)_3$ Cys-OH. This material is also commercially available from Boehringer Mannheim, Mannheim, FRG. For the synthesis of 2R palmitoyl and Troc contaning lipopeptide, the procedure of Kurimura et al [Peptide Chemistry 1990: Y. Shimonishi (Ed) Protein Research Foundation, Osaka, p 37-42 and 131-134 (1991)] is followed.

The lipopeptide - HIV annular antigen scaffold of this invention is not readily aqueous soluble. Thus, emulsions of this material may be prepared prior to administration, by Dounce homogenization, sonication or any other effective means. A dose of between about 1mg to 1 mg and preferably about 100-300 mg of the emulsified scaffold should be administered intramuscularly, subcutaneously or intravenously.

An alternate means for administering this lipophilic immunogen is as part of liposome or micelle. Any of a number of liposme forming lipids known in the art may be utilized for this purpose with the effect of carrying the scaffold of this invention into the biological system.

The scaffolds of this invention are administered as vaccines by incorporating the scaffold into a liposome, by adsorbing it onto aluminum hydroxide, or by first incorporating it into a liposome and then adsorbing the AAS/liposome to aluminum hydroxide. Alternatively, the scaffold is linked to a lipopeptide so as to yield a self-adjuvanting complex.

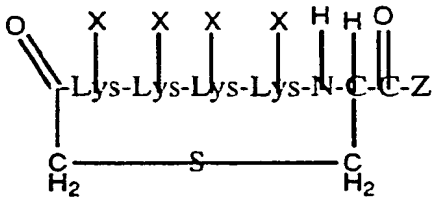
The antigenic scaffold is admininstered at a dose of between about 1μg to 1mg per kilogram. It may be administered subcutaneously, intravenously, or intramuscularly. The scaffold may be administered to a vertebrate to generate immune responses against the conjugated epitopes. Preferably, the vertebrate is a mammal, and most preferably, the mammal is a human. The immune responses generated

by presentation of the various antigenic epitopes on the AASC of this invention may be used in vitro. For example, the anti-HIV epitope antibody responses are useful in ELIZA assays or in BIAcore™ assays for characterization of HIV peptide binding affinities.

The following examples are provided to further define but not to limit this invention.

EXAMPLE 1

PREPARATION OF AN ANNULAR ANTIGEN SCAFFOLD CORE:



wherein:
S is a sulfur atom, contributed by the cysteine shown, forming a thioether bond;
X is $-NH_2$;
Z is ß-alanine-OH.

1. Synthesis of the linear peptide: Lys-Lys-Lys-Lys-Cys. [SEQ.ID:10:]:

Boc-ßAla-PAM Resin (Peninsula Labs, Lot 027925, 0.15 meq/g, 1 g) was washed three times with DCM, deprotected with 50% TFA/DCM for five minutes, and again for twenty minutes. The resin was washed again with DCM twice, five times with 5%DIEA, and then five more times with DCM.

Onto this resin, the active ester of N-Fmoc-S-Trityl-L-Cys [Bachem lot 2J541, 0.26g, prepared in NMP with BOP (0.19g) and NMM (0.45g, 0.49 mL)] was coupled to a negative ninhydrin. The amino terminus was then deprotected with 20% Piperidine in NMP followed by five NMP washes. Next, N-ε-Boc-N-α-Fmoc-L-Lys (Bachem lot 2J724, 0.211g) was prepared in NMP with BOP (0.19g) and NMM (0.45g, 0.49 mL) and was acylated onto the peptide to a

negative ninhydrin. An additional three cycles of lysine addition, following almost exactly the above procedure for addition of the first lysine, was then conducted, and the amino terminus deprotected by treatment with 20% piperidine in NMP for twenty minutes, followed by five NMP washes.

2. Cyclization of the linear peptide to form a thioether linked annular antigen scaffold core:

The deprotected amino terminus was bromoacetylated by addition of bromoacetic acid (Aldrich, AW03119ET, 0.45mmoles, 63 mg) plus dicyclohexylcarbodiimide (DCC, 0.225 mmoles, 46 mg) in DCM. This bromoacetylation was allowed to run for 1.5 hours and then repeated, by addition of the same reagents, to ensure complete bromoacetylation, for another 20 minutes.

The resin was washed five times with DCM. The Boc protection was removed using 50% TFA for 20 minutes, in the presence of 0.5 mL triethyl silane as a scavanger. Four DCM washes and one diethyl ether wash followed.

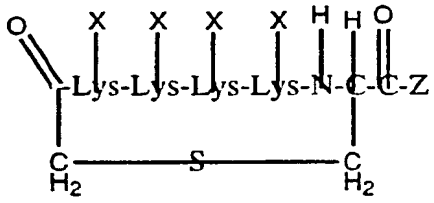
The peptide was cleaved from the resin in dry hydrofluoric acid (HF), without any scavanger, for one hour at 0°C. The HF was then evaporated. The peptide was separated from the resin by dissolution in TFA, filtering off the resin, and removing the TFA in a rotary evaporator. Cyclization of the peptide was allowed to occur by dissolving the peptide in 800 mL of double deionized water, and adding solid $NaHCO_3$ until the pH reached 8. The solution was stirred, and cyclization was allowed to proceed over approximately 48 hours. The liquid was then removed by rotary evaporation.

The peptide was dissolved in 8 mL of double deionized water and the cyclized AASC was recovered by loading onto a preparative Vydac C18 HPLC column at 3 mL/minute, and then eluting at 10 mL/minute using a gradient from 0-25% CH_3CN over ten minutes. Sample elution was monitored at 215 nm, with full scale deflection set at 2. Two peaks were collected and rechromatographed (2.5% CH_3CN-12.5% CH_3CN over 60 minutes) after drying in a rotary evaporator. A peak eluting at

23.4 minutes was collected and lyophilized, to yield 17.6 mg of peptide. The mass was analyzed by FAB-MS, and the predicted mass of 746 for the AASC was confirmed.

EXAMPLE 2

PREPARATION OF BROMOACETYLATED CORE:



wherein:
S is a sulfur atom, contributed by the cysteine shown, forming a thioether bond;
Z is ß-alanine-OH; and
X= bromoacetyl.

The HIV PND cyclic sulfides and other peptidyl epitopes may be conjugated to an AASC by bromoacetylating the free amino groups on the AASC and reacting the bromoacetylated groups with thiolated epitopes. For this purpose, the AASC is bromoacetylated as follows:

Bromoacetic anhydride, $(BrAc)_2O$ was prepared by dissolving 15.2 mg of bromoacetic acid (BrAcOH) in 2 mL of dry methylene chloride. Dicyclohexyl carbodiimide (DCC, 8 equivalents, 11.3 mg) was added and the mixture stirred at room temperature for one hour in the dark. At the same time, tetralysine cyclic core, TLCC (5.1 mg, 6.8 µmol) was dissolved in dry, degassed DMF. The $(BrAc)_2O$ was filtered through glass wool to remove dicyclohexylurea, DCU, and added to the solution of TLCC. After one hour, a 2µL aliquot was removed for Kaiser analysis which was negative. A second 2µL aliquot was removed, diluted with 100µL water and 10µL was injected into an analytical, reverse phase HPLC (RPHPLC, 1.5 mL/min,

Vydac C18, 10-60%CH_2CN over 20 minutes, λ=215nm, 0.1 abs. units full scale). Four peaks were observed.

An additional equivalent of $(BrAc)_2O$ (7.6 mg BrAcOH, 5.6 mg DCC) was prepared in 0.5 mL methylene chloride. This was filtered, added to the reaction mix, and the bromoacetylation was allowed to proceed overnight. When RPHPLC analysis showed no change after overnight reaction, the reaction was concentrated in vacuo and an additional two equivalents of $(BrAc)_2O$ in 500µL methylene chloride was prepared. The concentrated reaction was taken up in 1 mL of 1:1 DMF/methylene chloride, and the filtered $(BrAc)_2O$ was added. The reaction was stirred at room temperature for 5 hours. RPHPLC analysis revealed 3 major product peaks.

The reaction was concentrated in vacuo and products were isolated in a single RPHPLC run on a RCM25X10 C18 delta pak column with a gradient from 20-26% over 30 minutes at 10 mL/min. Four pooled fractions were collected, peaking at the indicated times: A-11 minutes; B-15 minutes; C-18 minutes; D-21 minutes. The C fraction was identified as having the correct mass of 5818 for tetrabromoacetylated product by electrospray mass spectrometry.

For conjugation of HIV PND cyclic sulfides and other epitopes with the bromoacetylated core, the epitope is thiolated by addition of a cysteine at the carboxy terminus. This is accomplished by coupling a cysteine protected at the SH by Trt and a tBu protection of the carboxyl in the presence of DCC and DCU. The protecting groups are then removed with 50% TFA and the free SH reacted with the bromoacetylated core.

EXAMPLE 3

PREPARATION OF CYCLIC HIV PND PEPTIDE EPITOPES:

Stably cyclic human immunodeficiency (HIV) principal neutralizing determinant (PND) peptides are produced by ring closure through a thioether linkage. The conserved sequence Gly Pro Gly Arg (SEQ. ID:1:) of the V3 loop of HIV is included in the sequence of these peptides, which may optionally include flanking aminoacides. Ring

closure is accomplished by bromoacetylation of the amino-terminus of synthetic peptides and allowing capture of a cysteine or homocysteine thiol residue incorporated near the carboxy-terminus of the peptide.

The linear peptide Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-Cys-Gly [SEQ.ID:2:] was prepared by standard methods of solid phase synthesis on a Milligen 9050 synthesizer, using 0.41 g of Fmoc-Gly-Wang Resin (Penninsula Labs Lot # 018347, 0.5 meq/g). The resin was mixed with three volumes of glass beads. Double couplings were conducted at Ile^3, Gly^4, Pro^5, Gly^6, and Arg^7. Piperidine (20%) was used to remove Fmoc at each cycle. Coupling was with the pentafluorophenyl amino acids.

At the end of synthesis, a ninhydrin reaction on the resin was dark blue. The peptide was bromoacetylated on the resin using paranitrophenylbromoacetate (0.25 g), DMAP (0.028 g) in NMP. The sample was washed with NMP and then dried. The peptide was cleaved/deprotected by addition of 90% TFA, 5 % thioanisole, 5 % thiocresol at room temperature for three hours.

The resin was filtered off and washed with 100% TFA. The combined filtrates were evaporated in vacuo to yield the crude peptide, which was washed with diethylether and dichloromethane to remove scavangers. The peptide was recovered by scraping and filtering (0.446 g).

Peptide (144.2 mg) was dissolved in water (600 mL) and the pH was raised to 8 by addition of sodium bicarbonate (1.45 g). The sample was stirred for twelve hours to allow cyclization to occur, and then dried by lyophilization.

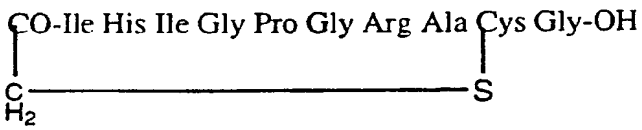
The cyclized peptide was dissolved in 20 % acetonitrile (12 mL). A yellow precipitate was removed by centrifugation prior to preparative HPLC (0-25% acetonitrile over 70 minutes, followed by isocratic elution at 25 % acetonitrile, in 2xC18 preparative columns in tandem, monitored at 215 nm, with 2 absorbance units full scale). The cyclized peptide eluted at 74 minutes. The sample was lyophilized (19.7 mg). An aliquot was measured by FAB-MS and gave the predicted mass of 1020.

Other cyclic HIV PND sulfides are prepared in essentially identical fashion to the method used above. Thus, the linear peptide Arg-Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-Phe-Tyr-Thr-Cys-Gly [SEQ.ID:3:] was prepared by standard solid phase synthesis using two couplings at Ile^2, Ile^4, Gly^5, Pro^6, Arg^8, Phe^{10}, Tyr^{11}. Sixty minutes was provided per cycle (90 minutes for the final arginine). Piperidine deprotection was allowed to run for ten minutes. FAB-MS gave the predicted 1588 for the cyclic sulfide.

EXAMPLE 4

PREPARATION OF AN AAS WITH CYCLIC HIV PND PEPTIDE ANTIGENS:

The cyclic HIV PND peptide, [SEQ. ID:2:]:



(24.2 mg, 23.7 µmoles) was added to a solution containing BOP (10.5 mg), NMM (3.5 mg, 4 µL), DMF (4 drops, dry). After 15 minutes, HOBt (3.2 mg), and AASC (2.2 mg) were added. DMF (9 drops, dry) was added and the mixture was sonicated for 30 minutes to a final bath temperature of 58°C. The mixture was then stirred for 15 minutes, sonicated for an additional 10 minutes, and then NMM (3 µL) was added and the mixture was stirred an additional 45 minutes. The DMF was removed by drying in a rotary evaporator. The conjugate was then solubilized in water (2 mL) and frozen.

The conjugate was thawed and purified by preparative HPLC using a gradient of 12.5% to 27.5% CH_3CN over 70 minutes. A peak eluting at about 25% CH_3CN was collected, and dried to yield 6.9 mg. This material, by electrospray mass spectrometry, gave the predicted mass of 4753.7 for AAS-1.

EXAMPLE 5

PREPARATION OF GnRH ANALOGS:

General: All reagents were used as received by the supplier. In the case of solvents, HPLC-grade was used where available. HPLC (binary gradient) was performed on a Waters 600E system with Waters 484 tunable U.V. detector (Aufs=0.1 analytical or 2.0 preparative scale) and recorded on a Waters 746 Data Module. A Waters WISP™712 autosampler (2000 mL sample loop) was used for analytical samples. A Rheodyne 7125 manual injection port (5000 mL sample loop) was used for preparative samples. A = H_2O, 0.1% TFA; B=CH_3CN, 0.1% TFA. Mass spectra were taken on a Finnegan MAT 90, spectrophotometer (positive ion, NBA matrix).

Abbreviations: Standard amino acid abbreviations are used. RT, room temperature; DCC, 1,3-dicyclo-hexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; DIEA, N,N-diisopropylethyl-amine; MPS, ß-maleimidopropionic acid N-hydroxy-succinimide ester; GnRH, gonadotropin releasing hormone; PBS, phosphate buffered saline; DTT, dithiothreitol; EDTA-2Na, ethylenediaminetetraacetic acid disodium salt; NBA, 3-nitro-benzyl alcohol.

6-D-Lys-GnRH,1:PyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2 [SEQ.ID:11:]:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (SPPS) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (2 h, RT) from the resin using reagent R (1 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC (Waters PrepPak®25 x 10™ C_{18}; 10 mL/min; 10-20% B, 0-20 min.; then 20-35% B, 20-40 min.; g=230 nm).

6-D-Lys-GnRH: FAB-MS (positive ion, NBA matrix) Calc. M+1 1254.44; Found M+1 = 1254.4

(N_e-maleimidopropanoyl)-6-D-Lys-GnRH,2:PyroGlu-His-Trp-Ser-Tyr-(N_e-maleimidopropanoyl)-D-Lys-Leu-Arg-Pro-Gly-NH_2 [SEQ.ID:12:]:

6-D-Lys-GnRH (10 mmol, 12.5 mg) was dissolved in N,N-dimethylformamide (0.5 mL/mg) and DIEA (50 mmol, 9 mL) added. The mixture was stirred briefly (RT) and ß-maleimidopropionic acid N-hydroxysuccinimide ester (MPS; 20 mmol, 5.2 mg) was introduced in one portion. After 30 min reaction time, 10 mL TFA was added to the reaction mixture and the solvent removed in vacuo. The peptide was purified by reverse phase HPLC (Waters PrepPak® 25 x 10™ Delta-Pak™ C_{18}; 10 mL/min; 10-25% B, 0-30 min.; then 25% B, 30-35 min; g=230 nm).

(N_e-maleimidopropanoyl)-6-D-Lys-GnRH,FAB-MS (positive ion, NBA matrix) Calc. M+1: 1405.56; Found M+1=1405.6

(N_e-Succinyl)-6-D-Lys-GnRH,2:PyroGlu-His-Trp-Ser-Tyr-(N_e-succinyl-D-Lys-Leu-Arg-Pro-Gly-NH_2 [SEQ.ID:12:]:

6-D-Lys-GnRH (7.98 µmol, 10 mg) was dissolved in N,N-dimethylformamide (1 mL) and DIEA (8 µmol, 8 µL) added, followed by succinic anhydride (1.6 mg, 16 µmol, 2 eq). The mixture was stirred for about 12 hours at room temperature. The solvent was removed in vacuo. The residue was dissolved in 0.1% TFA (1.4 mL) and purified by reverse phase HPLC (Vydac RCM8x 10 Delta-Pak™ C_{18}; 3 mL/min; 15-20% acetonitrile over30 min.). Combined product peaks were lyophilized (9.7 mg). An portion of the sample was analyzed by FAB-MS and the predicted mass for the succinylated product was confirmed.

(N_e-succinyl)-6-D-Lys-GnRH,FAB-MS (positive ion, NBA matrix) Calc. M+1: ; Found M+1=

Preparation of other GnRH Analogs

N-Ac-1,2-Di-p-Chloro-Phe-6-DLys-GnRH, Ac-4-Cl-Phe-4-Cl-Phe-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH_2 [SEQ.ID:13]:

The peptide is synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and double couplings (DCC/HOBT) for 4-Cl-Phe and single couplings for the remaining residues. The amino terminus is capped by treatment with acetic anhydride (5-10 mL) until the resin beads give a negative Kaiser test for the presence of an amine (0.5-8 h). The peptide is cleaved (2 h-4 h, RT) from the resin using reagent R (0.5 mL-3 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide is precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC and characterized by FAB-MS.

6-DLys-10-DAla-GnRH, H-Pgl-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-DAla-NH_2 [SEQ.ID:14:]:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thio-anisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 6-D-Lys-10-D-Ala-GnRH (positive ion, NBA matrix) Calc (m+1) = 1268.5; Found (m+1) = 1267.5.

6-DLys-9-Pro-NHEt-GnRH, H-Pgl-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt [SEQ.ID:15:]:

The peptide is synthesized on Oxime or Merrifield resin by solid phase peptide synthesis (Boc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide is cleaved (2 h-72 h, RT) from the resin with anhydrous ethyl amine. The crude protected peptide is precipitated with diethyl ether, collected by suction filtration, and dried overnight (over P_2O_5). The protecting groups are removed from the dry peptide by treatment with anhydrous HF (0°C, 0.5-2 h, 5-30 mL) in the presence of anisole (0.2-2 mL) and dimethyl phosphite (0.1-1 mL). The excess HF is removed in vacuo and the residue triturated with diethyl ether. The peptide is purified by preparative reverse phase HPLC and characterized by FAB-MS.

6-DOrn-GnRH, H-Pgl-His-Trp-Ser-Tyr-DOrn-Leu-Arg-Pro-Gly-NH_2 [SEQ.ID:16:]:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thio-anisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 6-D-Orn-GnRH (positive ion, NBA matrix)
Calc (m+1) 1239.4; Found (m+1) 1239.5.

3-Indolylpropionyl-6-DLys-GnRH,3-Indolylpropionyl-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH_2 [SEQ.ID:17:]:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings

(DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thio-anisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 3-indolylpropionyl-6-D-Lys-GnRH (positive ion, NBA matrix) Calc (m+1) 990.2; Found (m+1) 990.7.

3-Indolylpropionyl-6-DLys-9-Pro-NHEt-GnRH, 3-Indolyl-propionyl-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt [SEQ.ID:18:]:

The peptide is synthesized on Oxime or Merrifield resin by solid phase peptide synthesis (Boc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The 3-indolylpro-pionyl moiety is incorporated as N-formyl-3-indole-propionic acid. The peptide is cleaved (2 h-72 h, RT) from the resin with anhydrous ethyl amine. The crude protected peptide is precipitated with diethyl ether, collected by suction filtration, and dried overnight (over P_2O_5). The protecting groups are removed from the dry peptide by treatment with anhydrous HF (0°C, 0.5-2 h, 5-30 mL) in the presence of anisole (0.2-2 mL) and dimethyl phosphite (0.1-1 mL). The excess HF is removed in vacuo and the residue triturated with diethyl ether. The peptide is purified by preparative reverse phase HPLC and characterized by FAB-MS.

6-D-Cys-GnRH [SEQ.ID:19:]:

This peptide is prepared by solid phase synthesis as with any of the foregoing compounds with the addition of D-cysteine at the 6 position, as follows:

Rink amide resin (521 mg) was coupled sequentially with Gly, Pro, Arg, Leu, D-Cys, Tyr, Ser, Trp, His, Pyroglutamic acid. The Arg and Gly residues were double-coupled and the other residues were single-coupled. The resin was washed with methanol three times and dried under nitrogen (1.069 g resin = 0.539 g weight gain).

The peptide was cleaved for three hours, the resin was filtered off, and the peptide was dried under vacuum. The residue was triturated with diethyl ether and the precipitated peptide was collected by suction filtration and lyophilized (431.7 mg).

A portion of the peptide (195.5 mg) was dissolved in 0.1% TFA, 10% acetonitrile (3 mL), filtered, and purified by RPHPLC (15-45% acetonitrile over 30 minutes using two 25X10 RCM delta pak C18 columns in tandem. A repeat purification of a second portion of peptide was conducted (190.5 mg). Peak fractions were collected, combined and lyophilized, and an aliquot was analyzed by FAB-MS and amino acid analysis. The predicted mass (1229) and amino acid composition were confirmed.

6-D-Glu-GnRH [SEQ.ID:19:]:

This peptide is prepared by solid phase synthesis as with any of the foregoing compounds with the addition of D-glutamic acid at the 6 position, as follows:

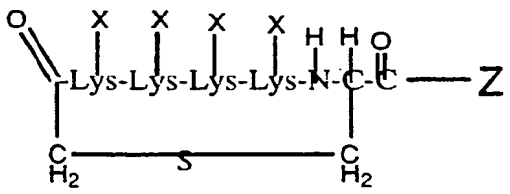
Rink amide MBHA resin (694 mg, 0.36 mmol/g) in an ABI 431A synthesizer was coupled sequentially with Gly, Pro, Arg, Leu, D-Glu, Tyr, Ser, Trp, His, Pyroglutamic acid. The Arg and Gly residues were double-coupled and the other residues were single-coupled. The resin was washed with DCM three times and dried under nitrogen (1.314 g resin).

The peptide was cleaved for three hours, the resin was filtered off, and the peptide was dried under vacuum. The residue was triturated with diethyl ether and the precipitated peptide was collected by suction filtration and lyophilized (200.8 mg).

The peptide was dissolved in 0.1% TFA, and purified by RPHPLC (10-60% acetonitrile over 30 minutes using two 25X10 RCM delta pak C18 columns in tandem, 13 mL/min) in two portions. Peak fractions were collected, combined and lyophilized (108.3 mg), and an aliquot was analyzed by FAB-MS and amino acid analysis. The predicted mass (1253) and amino acid composition were confirmed.

EXAMPLE 6

Preparation of GnRH Annular Antigen Scaffold Immunogen:



Summary:

Step 1 converts X from -NH_2 to bromoacetyl;
Z = ß-Ala-OH.

Step 2 converts X from bromoacetyl to an epitope linked scaffold, including GnRH peptides, HIV PND peptides, malaria antigens, or type-specific bacterial polysaccharides;
Z = ß-Ala-OH.

Step 3 converts Z to ß-Ala-NH_2

Step 4 acylates the ß-Ala-NH_2 with an adjuvant function.

Step 1: Preparation of Bromoacetylated Core:

Bromoacetic anhydride,$(BrAc)_2O$ was prepared by dissolving 58.6 mg (0.42 mmol) of bromoacetic acid (BrAcOH) in 3 mL of dry methylene chloride. Dicyclohexyl carbodiimide (DCC, 43.5 mg, 0.21 mmol) was added and the mixture stirred at room temperature for one hour in the dark. At the same time, annular antigen scaffold core, AASC, (26.2 mg, 35.1 µmol) was dissolved in dry, degassed DMF (3 mL). The $(BrAc)_2O$ was filtered to remove dicyclohexylurea, DCU, and added to the solution of AASC, and the reaction was stirred for one hour at room temperature. Kaiser analysis on an aliquot was negative.

The reaction was concentrated in vacuo and products were isolated in a single RPHPLC run on a delta prep 3000 using two RCM25X10 C18 delta pak columns with a gradient from 20-27% acetonitrile over 30 minutes at 15 mL/min. Three fractions were collected, peaking at the indicated times: A- 17 minutes; B- 19 minutes; C- 20 minutes. The B fraction was identified as having the correct mass of 1229 for tetrabromoacetylated product by fast atom bombardment mass spectrometry (FAB-MS).

Step 2: Linkage of GnRH to the Bromoacetylated Core:

Bromoacetylated core from step 1 (11.3 mg, 9.2 μmol, FW =1229) was dissolved in degassed nitrogen sparged pH 8 phosphate buffer (0.1 M, 2 mL), and stirred under an atmosphere of nitrogen. A 2μL aliquot was removed and diluted with 100μL of 0.1% aqeous TFA and saved as a reference. To the bromoacetylated core was added D-Cys^6-GnRH (45.2 mg, 36.8 μmol) and an additional 2 mL of buffer, followed by 500μL acetonitrile). The reaction was stirred overnight at room temperature. Analytical RPHPLC showed that there was no residual starting material. The reaction was therefore fractionated by preparative RPHPLC (RCM 25x10, 10 mL/minute, delta pak C18, 20-30%CH_3CN over 30 minutes, monitored at 230 nm with 1.25 absorbance units full scale). Product (12.3 mg) was collected and lyophilized. This material gave the correct mass of 5817 for a core having four D-Cys^6-GnRH peptides by FAB-MS.

Step 3. Provision of a free amine at the core's carboxy terminus:

The peptidylated core from step 2 (12.3 mg, 2.1 μmol) was dissolved in dry, degassed DMF. To the solution of peptidylated core was added diaminoethane-BOC (1.7 mg, 4.2 μmol) followed by DIEA (0.8 μL, 4.2 μmol) followed by bezotriazolyl-N-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate, BOP (1.9 mg, 4.2 μmol), and 1-hydroxybenzotriazole hydrate, HOBt, (0.6 mg, 4.2 μmol). The reaction was allowed to stir at room temperature and then at 4°C for 48 hours. The sample was concentrated in vacuo, and the

residue was taken up in aqueous 5% CH_3CN, filtered (0.45µm nylon filter) and fractionated by RPHPLC (RCM 25x10, 20-35% CH_3CN, over 30 minutes, 10 mL/min, monitored at 230 nm, 1.5 absorbance unit full scale, delta pak C18, 300 angstrom packing). The main peak was collected and lyophilized. By FAB-MS, the product had the correct mass of 5959.

The t-Boc product (7.3 mg, 1.22 µmol) was resuspended in methylene chloride (3 mL). Anhydrous anisole (1 mL) was added and the solution was stirred at 0°C. TFA (3 mL) was added and the solution stirred for forty minutes at 0°C and then allowed to reach ambient temperature over twenty minutes. The product was concentrated in vacuo, followed by lyophilization to remove all of the anisole, yielding 8.3 mg of product. The residue was dissolved in 0.1% TFA (3 mL). A 2 µL sample was analyzed in a Kaiser test which showed a faint blue color, confirming the presence of a free amino group appended to the antigen scaffold at the position Z. The product was lyophilized overnight (7.3 mg yield), which gave a mass of [INSERT MASS HERE] by electrospray mass spectrometry.

Step 4. Provision of an adjuvant function:

The free amino group provided in step 3 may be acylated with an adjuvant moiety. Thus, triacyl-glyceryl-cysteinyl-seryl-serine may be acylted to the free amine. $(Pam)_3Cys$-OH (173 mg, 0.19 mmole) is dissolved in NMP (1.0 mL) at 24°C slowly to give a clear, pale brown solution upon sonication and stirring. 4-Methyl morpholine (0.285 mmole) is added, followed by solid BOP (84 mg, 0.19 mmole) and HOBt (25.7 mg, 0.19 mmole). The mixture is again sonicated and stirred (5 min. 24°C) to obtain a clear solution. To the viscous solution is added more NMP (5 mL) and then mixed with the peptidylated AASC.

The thick reaction slurry is stirred at 24°C for 17 hours. A few resin particles are removed, washed sequentially with NMP, methylene chloride, and then air dried. Analysis by Kaiser test (ninhydrin reagent, Kaiser et al., Anal. Biochem. 34, 595-598 (1970))

gives a blue color indicating the presence of residual free amino groups. The acylation reaction is allowed to proceed for another 18 hours, at which point a solution of BOP (42 mg, 0.095 mmole), HOBt (12.8 mg, 0.095 mmole) and 4-methyl morpholine (0.143 mmole) is added in NMP (1 mL), and the reaction allowed to proceed further. At a negative Kaiser test, the reaction is complete and the product is isolated by HPLC as follows.

The material is triturated with ether (4x3 mL), and the insoluble product is recovered by filtration. The product is dried. A 0.46 x 25 cm VYDAC C_4 reverse phase column, heated electrically to 50°C is equilibrated with aq. 0.1% TFA/63% acetonitrile. An aliquot is dissolved by sonication in aq. 0.1% TFA/70% acetonitrile (0.1 mL), and injected. The injected sample is gradient eluted with aq. 0.1% TFA/63% - 70% acetonitrile over 30 minutes, and monitored at 215 nm, A = 3.00, 2.0 mL/min to isolate the product. A major peak is collected and concentrated by evaporation, followed by lyophilization. This same procedure is repeated with larger amounts of sample, and the eluted material is combined.

An inoculum is prepared by making an emulsion in physiologic saline at a final concentration of about 300 mg/ml. Alternatively, a liposome incorporating the hybrid at about 300 mg/ml may be prepared.

African green monkeys or rabbits are individually inoculated with three 300 µg doses or three 100 µg doses of the hybrid. The doses are delivered one month apart (week 0, 4 and 8). The animals are bled at intervals of two weeks. Serum samples are prepared from each bleed to assay for the development of GnRH specific antibodies. The same methods described above are equally applicable where different epitopes, such as HIV PND peptides, malaria peptides or type specific bacterial capsular polysaccharides are used in place of the GnRH peptides.

EXAMPLE 7

The biological activity of GnRH antigen scaffolds of this invention is demonstrated by biniding of the conjugate to pituitary membranes:

PITUITARY GnRH RECEPTOR BINIDING ASSAY

A. Preparation of pituitary membranes

1. Frozen rat pituitary glands were homogenized in 10 volume of ice cold buffer (Na_2HCO_3, 1 mM; dithiothreitol, 2.5 mM).
2. Centrifuged at 750 x g for 10 min.
3. Centrifuge the supernatant at 20,000 x g for 15 min.
4. Wash the pellet with Tris.HCl (20 mM, pH 7.4). Centrifuge at 20,000 x g for 15 min. Repeat washing one more time.
5. Resuspend the pellet in Tris.HCl buffer (140 mg fresh weight/ml).

B. Binding Assay

1. Membranes were incubated in a ice bath in 0.25 ml of medium (pH 7.5) containing 50 mM Tris.HCl, 1 mM dithiothritol, 0.1% BSA,
I^{-125} Buserelin (20,000 dpm) and desired concentration of test compound.
2. After 90-120 min. incubation, 5 ml of ice cold PBS (pH 7.5) were added to each tube and the content of tubes were rapidly filtered through a glass fiber filter which was been pre-soaked in 1% BSA in PBS. Wash the membrane on filter two times with 5 ml of cold PBS.
3. Determine the radioactivity of bound I^{-125} Buserelin in a gama counter. The IC_{50} of compounds for the inhibition of the I^{-125} Buserelin binding were estimated from competition curves.

C. Results:

The -[logIC50] of the scaffold of the instant invention, (6-D-Cys)4-(Lys)4-Cys-ß-Ala-OH, was measured according to this assay and found to be 8.25. The -[logIC50] for buserelin, 5-oxoPro-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-Pro-NH-C_2H_5 [SEQ.ID:20:], is 9.1, and that for natural GnRH is 7.8.

Thus, present invention provides a scaffold of GnRH antigens which have higher-affinity receptor binding activity than does natural GnRH and are thus functional receptor binding compounds. Thus, elicitation of immune responses by the conjugate of this invention results in depleteion in the circulatory system of active GnRH and therefore inhibition of the GnRH receptor.

EXAMPLE 8

Analysis of Sera for Anti-HIV cPND IgG Antibodies:

Each serum sample is analyzed by enzyme-linked immunoadsorbent assay (ELISA). Polystyrene microtiter plates are coated with 0.5 mg per well of the synthetic peptide in phosphate-buffered physiological saline (PBS) at 4°C. Each well is then washed with PBS containing 0.05% TWEEN-20 (PBS-T). Test serum, diluted serially in PBS-T, is added to the peptide-containing wells and allowed to react with the adsorbed peptide for one hour at 36°C. After washing with PBS-T, alkaline phosphatase-conjugated goat anti-human IgG is added to the test wells and allowed to react for one hour at 36°C. The wells are then washed extensively in PBS-T. Each well receives 0.1% p-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, containing 0.5 mM $MgCl_2 \cdot 6H_2O$. The ensuing reaction is allowed to proceed at room temperature for 30 minutes, at which time it is terminated by the addition of 3.0 N NaOH.

The greater the interaction of antibodies in the test serum with the peptide substrate, the greater is the amount of alkaline phosphatase bound onto the well. The phosphatase enzyme mediates the

breakdown of p-nitrophenyl phosphate into a molecular substance which absorbs light at a wavelength of 405 nm. Hence, there exists a direct relationship between the absorbance at 405 nm of light at the end of the ELISA reaction and the amount of peptide-bound antibody.

Similar assay are utilized to measure the affinity of antibodies generated in response to AASC linked to other eptiopes such as GnRH peptides, type-specific bacterial capsular polysaccharides, and malaria antigens.

EXAMPLE 9

Analysis of Sera for Activity which Specifically Neutralizes HIV Infectivity:

Virus-neutralizing activity is determined with an assay described by Robertson et al., J. Virol. Methods 20: 195-202 (1988). The assay measures specific HIV-neutralizing activity in test serum. The assay is based on the observation that MT-4 cells, a human T-lymphoid cell line, are readily susceptible to infection with HIV and, after a period of virus replication, are killed as a result of the infection.

The test serum is treated at 56°C for 60 minutes prior to the assay. This treatment is required to eliminate non-specific inhibitors of HIV replication. Heat treated serum, serially diluted in RPMI-1640 cell culture medium, is mixed with a standard infection dose of HIV. The dose is determined prior to the assay as containing the smallest quantity of virus required to kill all the MT-4 cells in the assay culture after a period of 7-8 days. The serum-virus mixture is allowed to interact for one hour at 37°C. It then is added to 1.0×10^5 MT-4 cells suspended in RPMI-1640 growth medium supplemented with 10% fetal bovine serum. The cultures are incubated at 37°C in a 5% CO_2 atmosphere for 7 days.

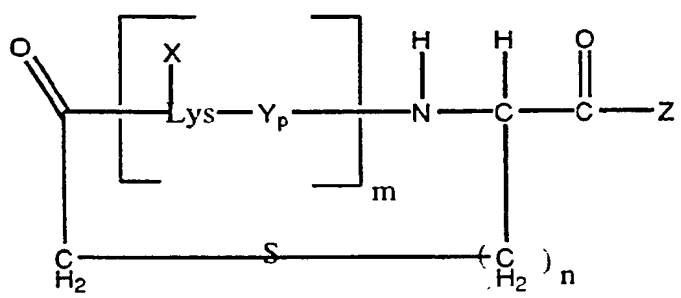
At the end of the incubation period, a metabolic dye, DTT, is added to each culture. This dye is yellow in color upon visual inspection. In the presence of live cells, the dye is metabolically processed to a molecular species which yields a blue visual color. Neutralized HIV cannot replicate in the target MT-4 cells and therefore

does not kill the cells. Hence, positive neutralization is assessed by the development of blue color following addition of the metabolic dye.

The annular antigen scaffold conjugates of HIV PND peptide of this invention are used to induce HIV neutralizing antibodies which are tested according to this method and are useful in identifying HIV PND peptides which bind with high affinity.

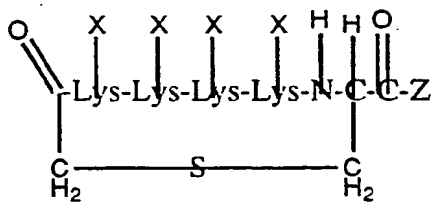
WHAT IS CLAIMED IS:

1. An annular antigen scaffold core (AASC) for antigens having the formula:



wherein:
S is a sulfur atom, forming a thioether bond;
X is an epitope with each X being linked through the α- or ε-amino group of lysine, with each X being the same or different;
Y is a linker;
Z is a moiety linked to the antigen scaffold, such as a peptide providing a T cell epitope, a lipopeptide which may provide an adjuvant effect, or another moiety performing a carrier function;
p is 0 to 5;
m is 3 to 10; and
n is 1 to 10.

2. The AASC of Claim 1 having the formula:

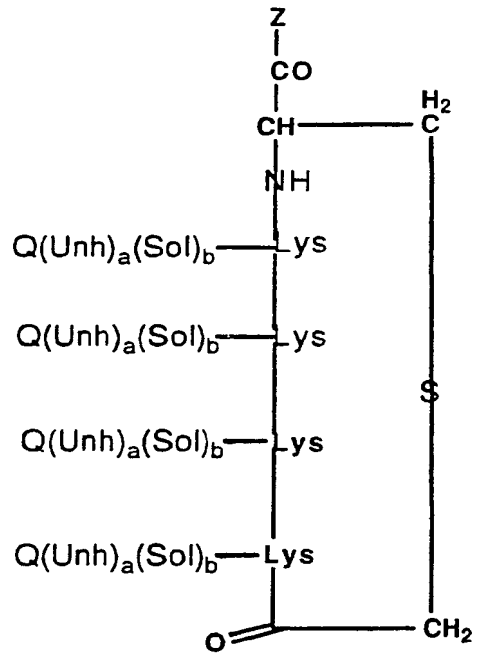


wherein:

S is a sulfur atom, contributed by the cysteine shown, forming a thioether bond;
X is an antigen with each X being the same or different;
Z is a moiety linked to the antigen scaffold, such as ß-alanine, a lipopeptide adjuvant, or another T-cell epitope.

3. The AASC of Claim 2 wherein the antigen is an HIV V3 loop peptide, a GnRH peptide, a malaria antigenic peptide, or a bacterial capsular polysaccharide.

4. An annular antigen scaffold having the formula:

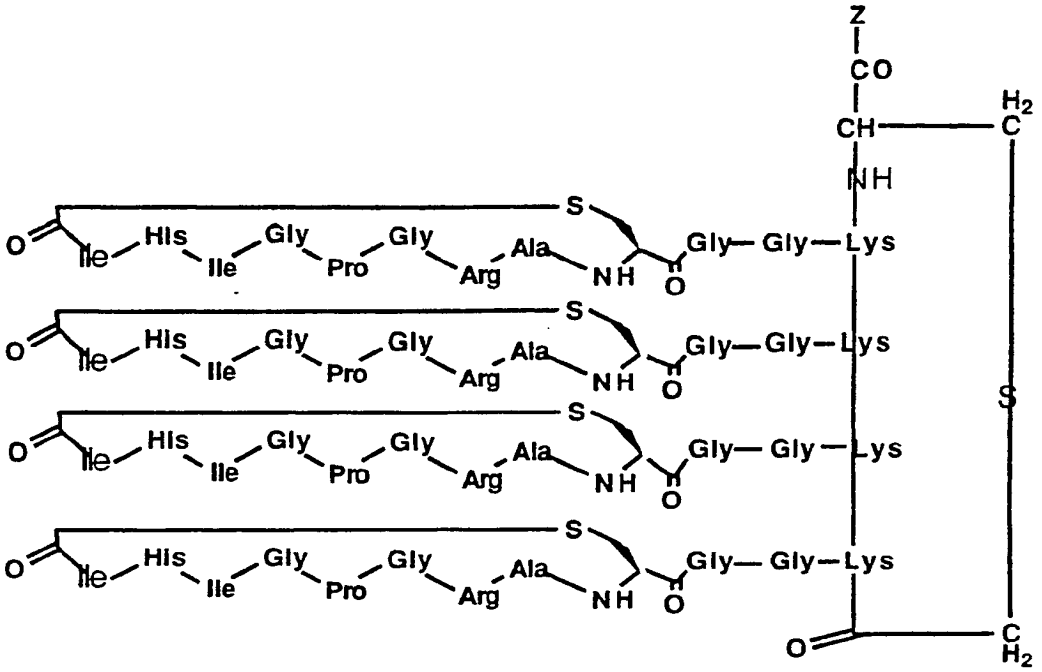


wherein:
Z is a moiety linked to the antigen scaffold, such as ß-alanine, a lipopeptide adjuvant, or another T-cell epitope;
Unh represents sterically unhindered groups;
Sol represents hydrophilic, solubilizing groups;

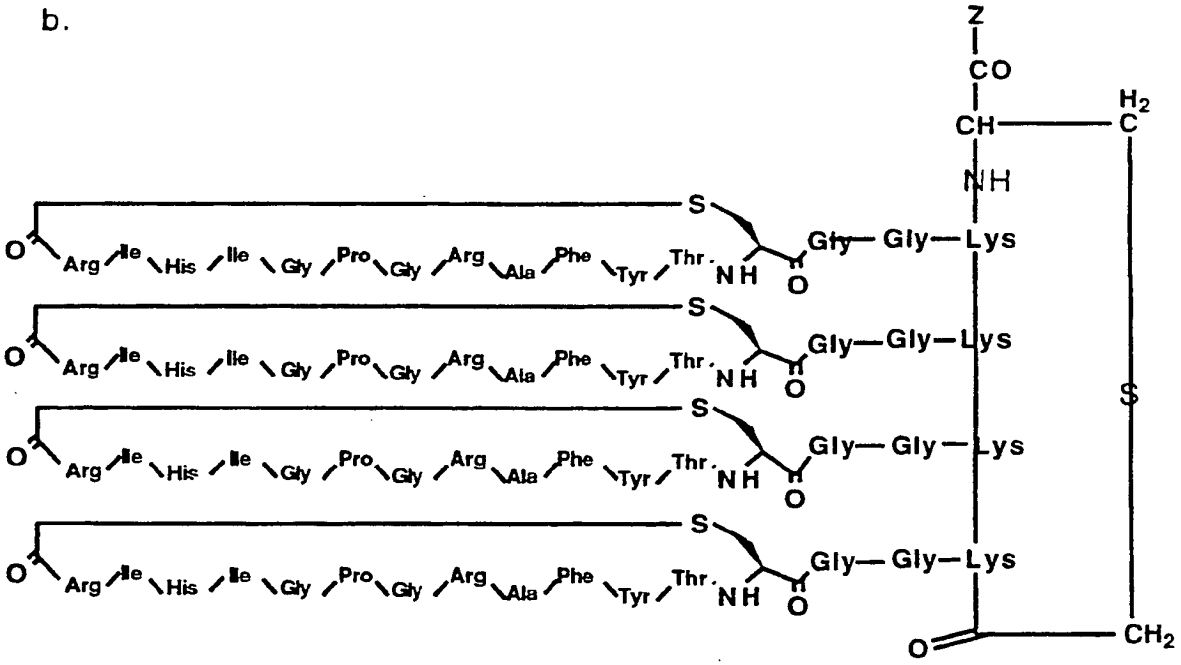
Q represents an antigen;
a is 0-5; and
b is 0-5.

5. An annular antigen scaffold having the formula:

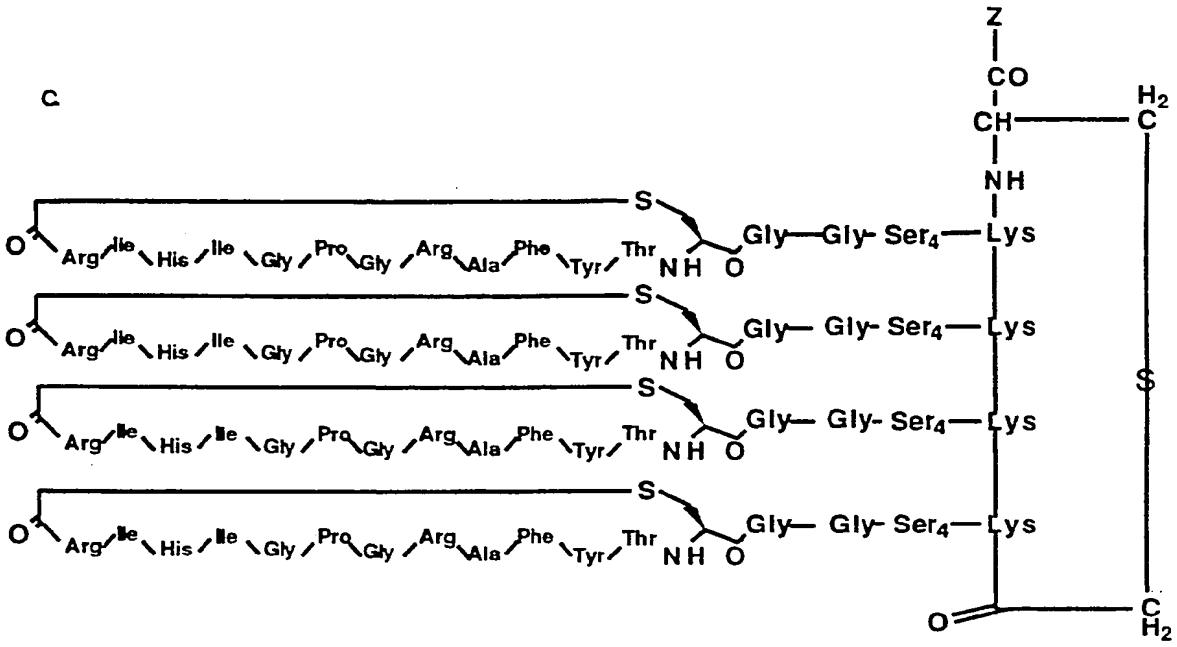
a.



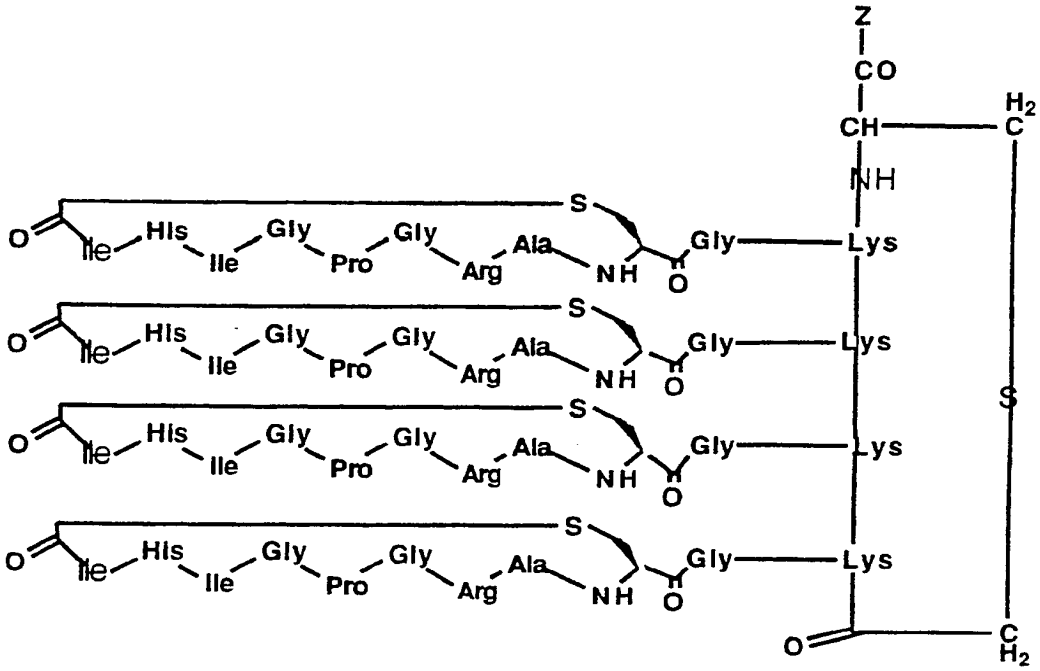
b.



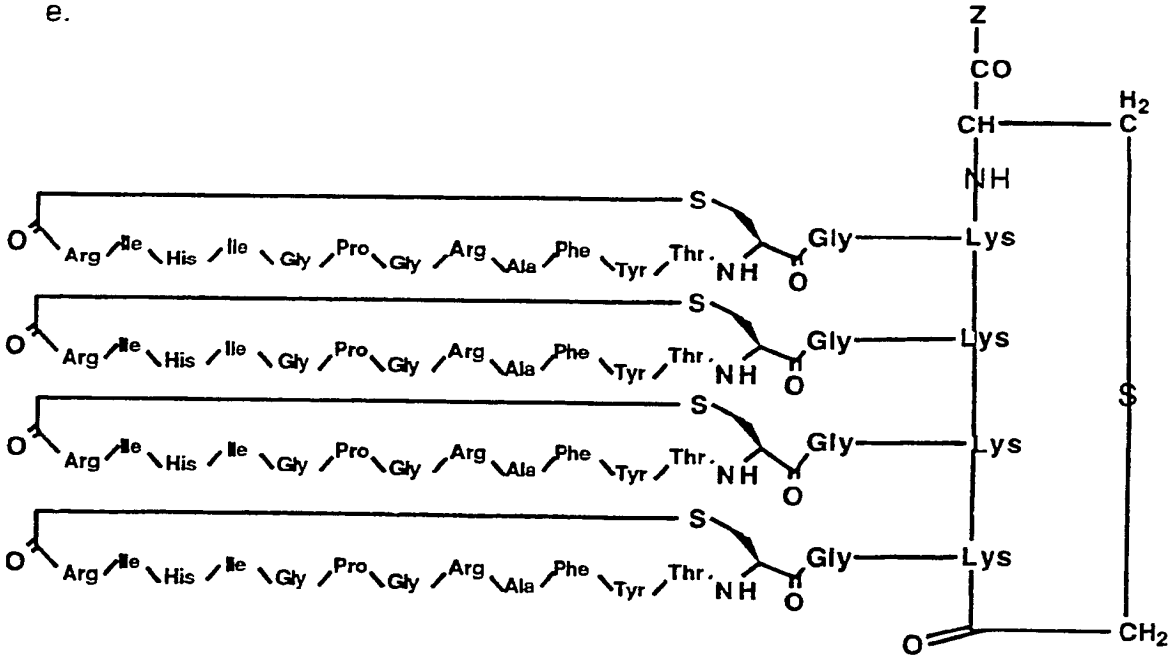
C.



d.



e.



6. A process for preparing an annular antigen scaffold core which comprises:

a) Synthesizing an annular multi-lysine core, referred to herein as an annular anitgen scaffold core (AASC), optionally incorporating solubilizing or sterically unhindered groups and purifying this component;

b) Isolating or synthesizing an antigen optionally incorporating a terminal groups of low steric hindrance and purifying this component; and

c) Coupling the purified core component of step (a) with the purified antigen of step (b), and purifying the AAS thus formed.

Patents Act 1977 Examiner's report to the Comptroller under Section 17 (The Search report) - 51 -	Application number GB 9420263.7
Relevant Technical Fields (i) UK Cl (Ed.M) C3H (HHX2, HA4) (ii) Int Cl (Ed.5) C07K 7/54, 7/64, 15/00, 15/12; A61K 39/00, 37/38, 39/21, 47/48 **Databases** (see below) (i) UK Patent Office collections of GB, EP, WO and US patent specifications. (ii) ONLINE DATABASES: WPI, CLAIMS, DIALOG/BIOTECH	Search Examiner C SHERRINGTON Date of completion of Search 9 JANUARY 1995 Documents considered relevant following a search in respect of Claims :- 1 to 6

Categories of documents

X:	Document indicating lack of novelty or of inventive step.	P:	Document published on or after the declared priority date but before the filing date of the present application
Y:	Document indicating lack of inventive step if combined with one or more other documents of the same category.	E:	Patent document published on or after, but with priority date earlier than, the filing date of the present application.
A:	Document indicating technological background and/or state of the art.	&:	Member of the same patent family, corresponding document.

Category	Identity of document and relevant passages	Relevant to claim(s)
A	EP 0328403 A2 (UNITED BIOMEDICAL INC) whole document	1, 4, 6
A	WO 92/18528 A1 (MEDICAL RESEARCH COUNCIL) whole document	1, 4, 6
A	Eur. J. Immunol. 1990, 20, 1619 - 1622 A multiple antigen peptide from the repetitive sequence of the Plasmoduim malariae...	1, 4, 6
A	Int. J. Peptide Protein Res. 1991, 37, 27 - 32 A new synthetic functionalized antigen carrier	1, 4, 6
A	J. Biol. Chem. 1988, 263, 1719 - 1725 A novel method for producing anti-peptide antibodies	1, 4, 6

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